

Identification and Characterization of the Protein Antigens of *Leptospira interrogans* serovar *hardjo*

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We radiolabeled *Leptospira* proteins with [³⁵S]methionine. Solubilized extracts of radiolabeled *L. interrogans* serovar *hardjo* strain hardjoprajitno were analyzed by one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis and fluorography. We compared the protein profile obtained in this manner to the protein profiles of various [³⁵S]methionine-labeled *Leptospira* spp. The profiles of the pathogenic *L. interrogans* strains were very similar but not identical and exhibited no obvious relationship to those of the two nonpathogenic species. We used solubilized, radiolabeled hardjoprajitno extracts and a sensitive radioimmunoprecipitation procedure to identify protein antigens recognized by immunoglobulin G antibodies present in various rabbit anti-*hardjo* sera. Homologous hyperimmune rabbit serum efficiently precipitated a large subset of proteins, the majority of which were between 30,000 and 66,500 daltons. Radioimmunoprecipitations with sera prepared against each of four recent *hardjo* isolates cultured from infected cattle produced similar results. Immunoprecipitations done with various radiolabeled *Leptospira* extracts and anti-hardjoprajitno serum demonstrated that the pathogenic leptospires possessed a number of cross-reactive major and minor protein antigens. By cell fractionation procedures, we found that most of the major protein antigens were present in the outer envelope. These proteins were exposed on the leptospiral cell surface because intact radiolabeled leptospires bound antibodies directed against them.

Leptospirosis is an acute, febrile, septicemic disease caused by the spirochetal species *Leptospira interrogans*. Pathogenic leptospires have a broad spectrum of hosts and are ubiquitous. Although not readily distinguishable on the basis of morphological, biochemical, and cultural characteristics, these organisms have distinct antigenic properties as demonstrated by agglutination and agglutinin-absorption tests. Such properties are important for serodiagnosis and provide the basis for classification by serovar. Approximately 180 known pathogenic serovars have been assembled into 19 serogroups on the basis of common cross-reacting agglutinogens (13).

Leptospires of the Hebdomadis serogroup, in particular serovar *hardjo*, are the most common agents of bovine leptospirosis in the United States as well as in Great Britain, New Zealand, Australia, Canada, and Argentina (19, 20, 22). Infections caused by these organisms are not readily diagnosed and are characterized by infertility, mastitis, abortion, and premature birth (20). Although mortality of adult animals is rare, high morbidity has contributed to significant economic losses.

Although leptospirosis is usually controlled by vaccination, recent studies have demonstrated a lack of complete protection within serovar *hardjo* and between serovars of the Hebdomadis serogroup (18). Because leptospiral vaccines usually consist of chemically inactivated whole cells, virtually nothing is known about the immunogenic components responsible for provoking a protective host immune response.

The purpose of this study was to identify and characterize the protein antigens of hardjoprajitno, the type strain of serovar *hardjo* commonly used for commercial vaccine production, and to compare them to four serovar *hardjo*

isolates recently cultured from naturally infected cattle. We radiolabeled *L. interrogans* with [³⁵S]methionine. This procedure permitted us to identify leptospiral protein antigens by radioimmunoprecipitation. In addition, we identified a number of cell surface-exposed (outer envelope) antigenic proteins. Because the *Leptospira* outer envelope has been shown to be a primary target for antibody-complement bactericidal reactions (1) as well as a potent immunogen (2, 4-7, 11), antibodies to these proteins may be an important component of the host immune response.

MATERIALS AND METHODS

Bacterial strains and medium. All *L. interrogans* serovars were obtained from the National Leptospirosis Reference Laboratory, National Animal Disease Center, Ames, Iowa. Serovar *hardjo* isolates designated T8515, T5279, and T5296 were cultured from kidney tissue samples taken from slaughtered cattle (19). Serovar *hardjo* isolate NISKU-3 was cultured from a urine sample taken from an infected cow.

L. biflexa serovar *patoc* strain Patoc I and *L. illini* 3055 (presently classified as *species incertae sedis*) were obtained from Nyles Charon, West Virginia University Medical Center, Morgantown.

All leptospires were grown in bovine serum albumin (BSA)-polysorbate 80 medium (10) at 30°C and passaged weekly into fresh medium. Growth was monitored with a Coleman photonephelometer by the method of Johnson and Harris (14).

Radiolabeling of *Leptospira* proteins with [³⁵S]methionine. All leptospires except the serovar *hardjo* isolates were grown in 10 ml of BSA-polysorbate 80 medium at 30°C with shaking to a density of 1.5×10^8 cells per ml. Leptospires were radiolabeled with 35 to 40 μ Ci of [³⁵S]methionine (Amersham Corp., Arlington Heights, Ill.) per ml until they attained a density of 2.5×10^8 cells per ml. After being

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radiolabeled, the organisms were pelleted by centrifugation at $20,000 \times g$ for 15 min at 4°C and washed twice with 10 ml of BSA-polysorbate 80 medium (without albumin). A variety of detergents and conditions were used to solubilize the leptospire for gel electrophoresis and immunoprecipitation (see below). The best results were obtained by solubilizing the cells in 10 mM Tris (pH 7.5)–1 mM EDTA–1% sodium dodecyl sulfate (SDS). The extracts were heated for 3 min in a boiling water bath and centrifuged for 10 min in a microfuge at room temperature. Supernatants containing the radiolabeled proteins were removed and stored at -20°C .

Serovar *hardjo* isolates were radiolabeled in a similar manner, except that [^{35}S]methionine was added to 2×10^7 cells per ml, and solubilized cell extracts were prepared when the leptospire attained a density of 7×10^7 cells per ml.

Rabbit sera. All New Zealand White rabbits used for the production of antisera were bled before immunization to obtain normal sera. These sera were negative for the presence of leptospiral antibodies as determined by the microscopic agglutination test with live antigen (9). Rabbit anti-*Leptospira* serum was prepared by injecting rabbits intravenously with 2×10^8 leptospire per ml from a semisolid culture on day 0, 4×10^8 leptospire per ml on day 7, and 6×10^8 leptospire per ml on day 14. Sera from blood collected on day 21 were tested by microscopic agglutination with the homologous *Leptospira* strain as the antigen. Animals with microscopic agglutination titers of 1:5,000 or greater were exsanguinated by cardiac puncture. Sera were filter sterilized and then lyophilized or stored frozen.

Immunoprecipitation of radiolabeled *Leptospira* proteins. Solubilized radiolabeled *Leptospira* extracts (5 to 10 μl) were mixed with 500 μl of Triton buffer (17) and 15 μl of normal or test rabbit serum. After overnight incubation at 4°C , 100 μl of a slurry of protein A-Sepharose CL-4B (Sigma Chemical Co., St. Louis, Mo.) was added. The mixture was incubated at 4°C for 60 min with gentle agitation, pelleted, and washed three times in Triton buffer and three times in 10 mM Tris (pH 8.0). The immunoprecipitate was extracted from the pellet by adding 100 μl of SDS sample buffer (3), heating in a boiling water bath for 2.5 min, and centrifugation in a microfuge for 10 min. A supernatant containing the precipitate was collected and stored at -20°C .

Cell fractionation. Approximately 3×10^9 cells of serovar *hardjo* strain hardjoprajitno radiolabeled with [^{35}S]methionine were pelleted by centrifugation, washed twice in BSA-polysorbate 80 medium (without albumin), and suspended in 2 ml of 10 mM Tris (pH 8.0)–1 mM EDTA–2 mM phenylmethylsulfonyl fluoride. Leptospire were disrupted by sonication (Fisher model 300; five 5-s bursts with Micro-tip at 30% power). DNase (Sigma) and 200 μl of 2.5 mM MgCl_2 were added to the sonically treated material. After incubation at 4°C for 30 min, unlysed cells were removed by centrifugation in a microfuge for 15 min at 4°C . A 0.5-ml sample representative of the whole sonic extract was removed and processed as described below. Soluble and membrane proteins were separated by ultracentrifugation at $100,000 \times g$ for 90 min at 4°C . The pellet (membrane fraction) was suspended in 1.5 ml of 10 mM Tris (pH 8.0)–0.5 M KCl by homogenization. Ultracentrifugation of the membrane and soluble fractions was repeated. The membrane fraction was solubilized in 300 μl of 10 mM Tris (pH 7.5)–1 mM EDTA–1% SDS. Proteins in the soluble fraction and the whole sonic extract were precipitated by mixing with an equal volume of cold 10% trichloroacetic acid, incubation on ice for 15 min, and centrifugation in a microfuge for 2 min.

Precipitates were washed once with cold acetone. The soluble protein fraction was solubilized in 300 μl of 10 mM Tris (pH 7.5)–1 mM EDTA–1% SDS. The whole sonic extract proteins were solubilized in 100 μl of the same buffer. Each of the solubilized fractions was processed as described for whole cells.

Detection of cell surface-exposed antigenic proteins. A modification of the whole-cell radioimmunoprecipitation method of Hansen et al. (12) was used to identify antibody-accessible proteins on the cell surface of serovar *hardjo* strain hardjoprajitno. Briefly, [^{35}S]methionine-labeled intact leptospire were washed twice with 10 ml of BSA-polysorbate 80 medium (without albumin) and suspended in the same medium to approximately 6×10^8 cells per ml. Equal amounts (50 μl) of heat-inactivated normal rabbit serum, rabbit anti-hardjoprajitno serum, rabbit anti-hardjoprajitno serum exhaustively absorbed with intact leptospire, or phosphate-buffered saline were added to separate 1-ml portions of cells, and the mixtures were gently agitated at 4°C for 90 min. Leptospire were then pelleted by centrifugation and washed twice in BSA-polysorbate 80 medium (without albumin). Antigen-antibody complexes were extracted in solubilization buffer (12) for 60 min at 37°C . The extracts were centrifuged at 4°C for 15 min in a microfuge to remove insoluble material. A 200- μl volume of each extract was mixed with 100 μl of a slurry of protein A-Sepharose CL-4B and then incubated at 4°C for 60 min with gentle agitation. The protein A-antibody-antigen complexes were pelleted, washed five times with solubilization buffer and twice with 10 mM Tris (pH 8.0). Immunoprecipitations were extracted as previously described.

Preparations of radiolabeled *Leptospira* outer envelope. The outer envelope of serovar *hardjo* strain hardjoprajitno was obtained by a modification of the procedure of Auran et al. (2). Approximately 6×10^9 leptospire radiolabeled with [^{35}S]methionine as previously described were mixed with 6×10^{10} unlabeled leptospire. The addition of the unlabeled cells was necessary for visualization of the outer envelope pellet. The leptospire were pelleted by centrifugation, washed twice in BSA-polysorbate 80 medium (without albumin), and suspended in 2 ml of distilled water. A 30-ml volume of 1 M NaCl was immediately added, and the mixture was incubated for 50 min at room temperature to allow the formation of salt-altered cells, in which the outer envelope had dissociated from the protoplasmic cylinder. The salt-altered cells were pelleted at $20,000 \times g$ for 20 min at 4°C and suspended in 2 ml of distilled water. Addition of a 30-ml volume of 0.04% SDS solubilized the outer envelope and released the protoplasmic cylinder, thus converting the salt-altered cells to spiral cells. Protoplasmic cylinders were pelleted by centrifugation. The supernatant containing the outer envelope was centrifuged a second time to remove any residual protoplasmic cylinders. The resultant supernatant was subjected to ultracentrifugation at $100,000 \times g$ for 90 min at 4°C . The pelleted outer envelope proteins were homogenized in 600 μl of 10 mM Tris (pH 7.5)–1 mM EDTA–1% SDS and processed as previously described.

SDS-PAGE and fluorography. The SDS-polyacrylamide gel electrophoresis (PAGE) system used has been described previously (3). Radiolabeled cell extracts and immunoprecipitates were electrophoresed on 16-cm 15% acrylamide slab gels. The gels were stained with Coomassie blue, destained, and processed for fluorography as previously described (17). Molecular weights were determined based on the position of unlabeled known protein standards (Bio-Rad Laboratories, Richmond, Calif.).

RESULTS

Radiolabeling of *Leptospira* strains with [³⁵S]methionine and comparison of the protein profiles by SDS-PAGE. *L. interrogans* serovar *hardjo* strain hardjoprajitno cells radiolabeled with [³⁵S]methionine as described above were analyzed by one-dimensional SDS-PAGE and fluorography (Fig. 1). A complex array of protein bands ranging from approximately 14,400 to >92,000 daltons was readily discernible. Comparison of the protein profile obtained in this manner to the Coomassie blue-stained protein profile indicated that virtually a full complement of strain hardjoprajitno proteins was radiolabeled (data now shown). We found that by using the same radiolabeling protocol we could efficiently and reproducibly radiolabel all *Leptospira* strains and isolates used in this study. Figure 1 shows a direct comparison of the protein profiles obtained by SDS-PAGE and fluorographic analysis of solubilized extracts prepared from various [³⁵S]methionine-labeled *Leptospira* strains. We observed that the four recent isolates of serovar *hardjo* cultured from naturally infected cattle exhibited nearly identical protein profiles. The protein profile of strain hardjoprajitno was for the most part similar to that of the *hardjo* isolates, although some differences were discernible. We also compared the protein profiles of serovar *hardjo* leptospire to those of serovar *pomona* and serovar *balcanica*, another member of the Hebdomadis serogroup. The protein profiles of these two serovars were basically identical and appeared to be similar to the profile of strain hardjoprajitno. Finally, a comparison of the protein profiles of the pathogenic leptospire with those exhibited by two nonpathogenic species (Fig. 1) indicated that there was no obvious relationship between these organisms and any of the *L. interrogans* strains examined.

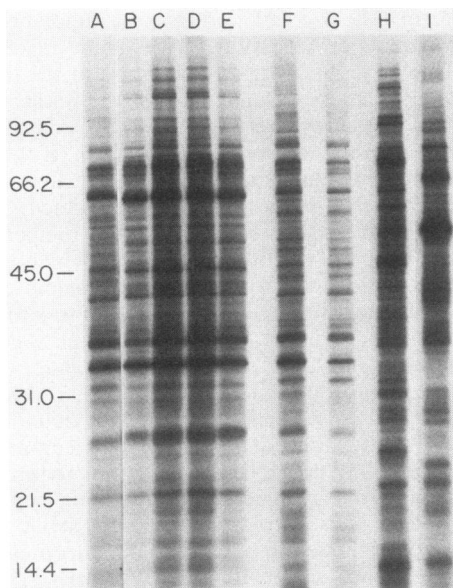


FIG. 1. Comparison of radiolabeled protein profiles of various *Leptospira* spp. by SDS-PAGE and fluorography. Leptospire were radiolabeled with [³⁵S]methionine, washed, solubilized, and analyzed by SDS-PAGE and fluorography as described in the text. Lanes: A, *L. interrogans* serovar *hardjo* strain hardjoprajitno; B, *hardjo* isolate T8515; C, *hardjo* isolate NISKU-3; D, *hardjo* isolate T5279; E, *hardjo* isolate T5296; F, serovar *pomona* isolate T5289; G, serovar *balcanica* isolate E32; H, *L. biflexa* serovar *patoc* strain Patoc I; and I, *L. illini* 3055. All samples were run on the same gel. Kilodalton markers appear on the left.

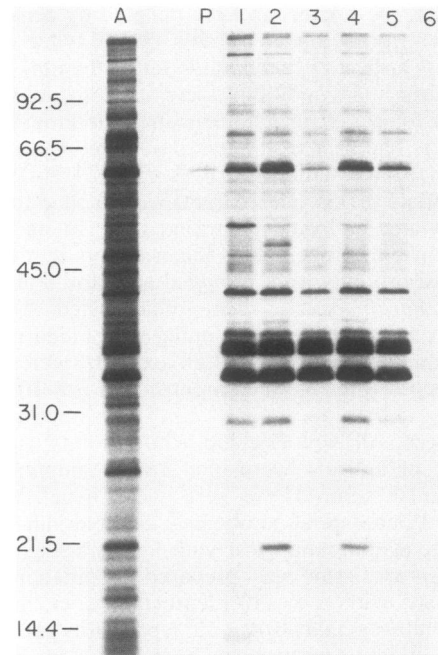


FIG. 2. Radioimmunoprecipitation of serovar *hardjo* strain hardjoprajitno protein antigens with various anti-*hardjo* sera. Immunoprecipitations with solubilized radiolabeled strain hardjoprajitno extract, rabbit serum, and protein A-Sepharose CL-4B were performed as described in the text. Precipitates were analyzed by SDS-PAGE and fluorography. Lane A, solubilized whole-cell strain hardjoprajitno antigen extract. Precipitates were obtained with pooled normal rabbit serum (lane P), high-titer serum from rabbits immunized with strain hardjoprajitno (lane 1), *hardjo* isolate T8515 (lane 2), NISKU-3 (lane 3), T5279 (lane 4), and T5296 (lane 5). Lane 6 shows the precipitates obtained in the absence of serum. Kilodalton markers appear on the left.

Protein antigens of strain hardjoprajitno identified by radioimmunoprecipitation. The availability of radiolabeled leptospiral proteins allowed us to use a highly sensitive radioimmunoprecipitation procedure to identify protein antigens recognized by immunoglobulin G antibodies present in various anti-*hardjo* and control sera. Radiolabeled, solubilized strain hardjoprajitno extracts were prepared, and the protein antigens were precipitated with rabbit sera, using protein A-Sepharose CL-4B. Precipitates were analyzed by SDS-PAGE and fluorography. We found that normal rabbit serum weakly precipitated one protein of approximately 63,000 daltons (Fig. 2). High-titer rabbit anti-*hardjoprajitno* serum prepared by intravenous injection of whole cells efficiently precipitated many leptospiral proteins, the majority of which were between 30,000 and 66,500 daltons. Similar results were obtained with sera from two other rabbits immunized in the same manner (data not shown). Radioimmunoprecipitations were also performed with strain hardjoprajitno extract and rabbit sera prepared against each of the four recent *hardjo* isolates. There were some minor differences in the protein antigens of strain hardjoprajitno recognized by the heterologous sera (Fig. 2). These differences probably reflect the minor differences we observed in the respective protein profiles (Fig. 1). However, for the most part the heterologous sera recognized the same major protein antigens that were recognized by immunoglobulin G antibodies present in the homologous serum.

Cross-reactivity of strain hardjoprajitno protein antigens

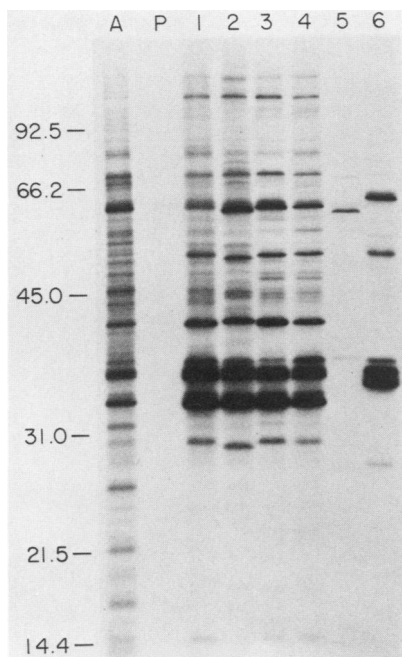


FIG. 3. Radioimmunoprecipitation of various *Leptospira* cross-reacting protein antigens with rabbit anti-hardjoprajitno serum. Immunoprecipitations were performed with solubilized, radiolabeled *Leptospira* extracts, rabbit serum, and protein A-Sepharose CL-4B. Precipitates were analyzed by SDS-PAGE and fluorography. Lane A, solubilized whole-cell strain hardjoprajitno antigen extract. Lane P, precipitate obtained with pooled normal rabbit serum. The remaining precipitates were obtained with anti-hardjoprajitno serum and the following extracts (lanes): 1, strain hardjoprajitno; 2, *hardjo* isolate T8515; 3, *pomona* isolate T5289; 4, *balcanica* isolate E32; 5, *L. biflexa* serovar Patoc; and 6, *L. illini*. Kilodalton markers appear on the left.

with other *Leptospira* protein antigens. We used the radioimmunoprecipitation procedure to further investigate the cross-reactivity of the protein antigens of strain hardjoprajitno with other *Leptospira* protein antigens. Radiolabeled leptospiral antigen extracts were prepared, and cross-reactive protein antigens were precipitated with rabbit anti-hardjoprajitno serum and then analyzed by SDS-PAGE and fluorography as previously described. A comparison of the radioimmunoprecipitation profile of strain hardjoprajitno and the radioimmunoprecipitation profile of *hardjo* isolate T8515 or serovars *pomona* and *balcanica* indicated that most of the major protein antigens as well as several minor protein antigens contained common antigenic determinants (Fig. 3). Note that for *hardjo* isolate T8515, the slight differences in the molecular weights of some of the protein antigens when compared with the respective hardjoprajitno protein antigens were a reflection of the minor differences that we observed in the protein profiles of these two organisms (Fig. 1). In contrast to the results obtained with the pathogenic *Leptospira* strains, only one protein antigen of the nonpathogenic *L. biflexa* serovar *patoc* and five to six antigens of *L. illini* were readily immunoprecipitated with anti-hardjoprajitno serum. These results were not unexpected in view of the dissimilarity between the protein profiles of these two organisms and that of strain hardjoprajitno (Fig. 1). Furthermore, these results illustrate the specificity of our radioimmunoprecipitation procedure.

Soluble and membrane-bound proteins of strain hardjoprajitno. [³⁵S]methionine-labeled strain hardjoprajitno lep-

tospire were disrupted by sonication, and the membrane fraction was separated from the soluble proteins by ultracentrifugation as described above. Proteins present in the total sonic extract and each fraction were analyzed by SDS-PAGE and fluorography (Fig. 4). We found that the protein profile exhibited by the total sonic extract was virtually identical to that of leptospire solubilized in SDS. The soluble and membrane fractions exhibited unique protein profiles. Some proteins appeared to be present in both fractions. These proteins may be comigrating species that were previously obscured in the one-dimensional protein profile of whole, solubilized leptospire, or they may be peripheral membrane proteins that were partially removed from the membrane when the cells were disrupted. Note that a careful comparison of Fig. 2 and 4 indicates that most of the major protein antigens immunoprecipitated by rabbit anti-hardjoprajitno serum were present in the membrane fraction.

Cell surfaced-exposed antigenic proteins. We used a modification of a whole-cell radioimmunoprecipitation procedure described by Hansen et al. (12) to identify antibody-accessible proteins on the cell surface of strain hardjoprajitno. Leptospire radiolabeled with [³⁵S]methionine were washed and then incubated for 1 h with normal rabbit serum, high-titer rabbit anti-hardjoprajitno serum, anti-hardjoprajitno serum exhaustively absorbed with intact leptospire, or buffer. Unbound antibodies were removed by washing. Leptospire were solubilized and antigen-antibody complexes were precipitated with protein A-Sepharose CL-4B as described above. The results appear in Fig. 5. Normal rabbit serum recognized the same 63,000-dalton protein that it previously recognized in the radioimmunoprecipitation of whole, solubilized leptospire (Fig. 2). Additionally, normal rabbit serum weakly recognized two proteins (41,500 and 21,500 daltons) that were also weakly precipitated in the

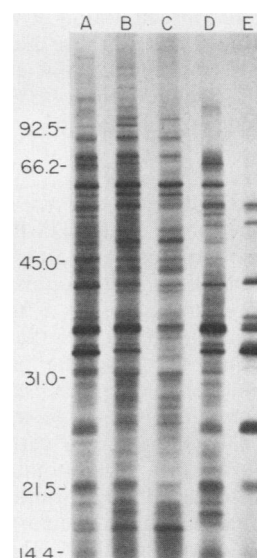


FIG. 4. Fractionation of [³⁵S]methionine-labeled strain hardjoprajitno cells. Soluble, membrane-bound, and outer envelope proteins of radiolabeled leptospire were prepared as described in the text and analyzed by SDS-PAGE and fluorography. Lanes: A, whole cells solubilized in buffer containing 1% SDS; B, whole-cell sonic extract; C, soluble protein fraction; D, membrane protein fraction; E, outer envelope protein fraction. Kilodalton markers appear on the left.

absence of serum, indicating some minor nonspecific binding to protein A-Sepharose CL-4B. On the other hand, anti-hardjoprajitno serum efficiently recognized at least nine distinct protein bands (63,000, 55,000, 51,500, 41,500, 38,000, 36,000, 35,500, 33,000, and 21,500 daltons). Each of these proteins corresponds to a specific membrane protein that was identified by the cell fractionation procedure (Fig. 4). Note that when we used anti-hardjoprajitno serum exhaustively absorbed with intact leptospire, most of the previously recognized proteins were either no longer precipitated or less efficiently precipitated, clearly indicating that these protein antigens were exposed on the leptospiral cell surface (Fig. 5).

Identification of *Leptospira* outer envelope antigenic proteins. To determine which membrane proteins reside in the leptospiral outer envelope, we used a modification of a procedure described by Auran et al. (2). Briefly, radiolabeled hardjoprajitno cells were treated with NaCl, allowing for the dissociation of the outer envelope-cell wall complex. The outer envelope was solubilized with SDS, cell cylinders were removed by centrifugation, and envelope proteins were pelleted by ultracentrifugation as described above. We found that the outer envelope proteins represented a subset of the membrane proteins and were composed of approximately nine major protein bands (Fig. 4, lane E). A comparison of Fig. 4 and 5 indicated that the outer envelope proteins correspond to the same proteins identified in the whole-cell radioimmunoprecipitation procedure as cell surface-exposed antigenic proteins. An additional protein of approximately 25,500 daltons not identified by this procedure because of lack of reactivity with anti-hardjoprajitno serum was also clearly an outer envelope protein. We found that solubilized radiolabeled extracts of strain hardjoprajitno outer envelope proteins were readily immunoprecipitated with the rabbit serum against any of the four serovar *hardjo* isolates or the serovar *pomona* and *balcanica* isolates used in this study (data not shown). Thus, as previously indicated in the results obtained by radioimmunoprecipitation of whole, solubilized leptospiral extracts (Fig. 2 and 3), these cell surface-exposed proteins of strain hardjoprajitno exhibit antigenic cross-reactivity with the cell surface proteins of the other pathogenic *Leptospira* strains that we examined.

DISCUSSION

In this study we radiolabeled *Leptospira* proteins with [³⁵S]methionine. A one-dimensional SDS-PAGE comparison of the radiolabeled protein profile of hardjoprajitno, the type strain of serovar *hardjo*, to the Coomassie blue-stained protein profile indicated that virtually a full complement of leptospiral proteins was labeled under the conditions used. We compared, for the first time, the protein profiles of various [³⁵S]methionine-labeled *Leptospira* strains. The profiles of four recent individual isolates of serovar *hardjo* that were cultured from naturally infected cattle were basically identical. The profile of strain hardjoprajitno was similar to that of the *hardjo* isolates, although some differences were apparent. These results are of particular interest because Robinson et al. (16) demonstrated by DNA restriction endonuclease analysis that field isolates of serovar *hardjo* are indistinguishable from one another but differ from strain hardjoprajitno. We found that two additional pathogenic serovars, *pomona* and *balcanica*, exhibit protein profiles that are very similar to strain hardjoprajitno. Based on serological tests, serovars *balcanica* and *hardjo* are known to be antigenically similar and have often been difficult to differentiate (15). Some antigenic relatedness between

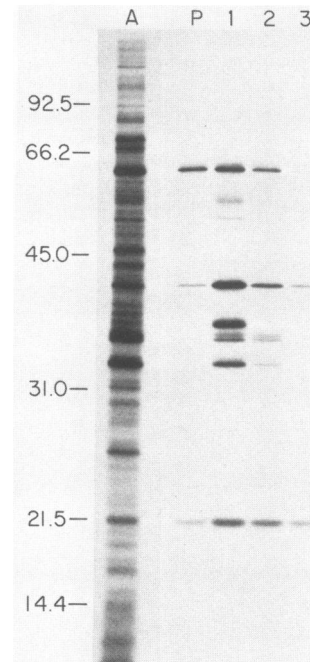


FIG. 5. Radioimmunoprecipitation of strain hardjoprajitno cell surface-exposed protein antigens. Intact, radiolabeled leptospire were incubated with normal rabbit serum, anti-hardjoprajitno serum, anti-hardjoprajitno serum absorbed with whole cells, or buffer. Unbound antibodies were removed by washing, and antigen-antibody complexes were solubilized and precipitated with protein A-Sepharose CL-4B. Precipitates were analyzed by SDS-PAGE and fluorography. Lane A, whole radiolabeled leptospire solubilized in the buffer of Hansen et al. (12). Precipitates were obtained with normal rabbit serum (lane P), anti-hardjoprajitno serum (lane 1), absorbed anti-hardjoprajitno serum (lane 2), or buffer (lane 3). Kilodalton markers appear on the left.

pomona and *hardjo* has also been observed (21). A comparison of the protein profiles of the three pathogenic serovars with those of the two nonpathogenic (i.e., free-living) species, *L. biflexa* and *L. illini*, indicated that there is no relationship between these organisms. Such results were not unexpected, because studies by Brendle et al. (8) involving DNA-DNA hybridization have shown that *L. interrogans* strains are genetically unrelated to *L. biflexa* or *L. illini*. These two organisms present unique protein profiles and are also genetically unrelated (8).

We used solubilized extracts of radiolabeled *Leptospira* strains and a sensitive radioimmunoprecipitation procedure to identify protein antigens recognized by immunoglobulin G antibodies present in various hyperimmune rabbit sera. For these experiments, as well as many of the subsequent experiments, we chose to work with strain hardjoprajitno because it grows to a higher cell density than the more fastidious *hardjo* isolates. We found that homologous serum specifically precipitated a large subset of leptospiral proteins. Heterologous sera prepared against each of the four recent *hardjo* isolates produced similar results. The high degree of cross-reactivity between these leptospire was not surprising in view of the resemblance between the protein profiles of these organisms. To further investigate the cross-reactivity of the protein antigens of strain hardjoprajitno, immunoprecipitations were performed with various solubilized radiolabeled *Leptospira* extracts and anti-hardjoprajitno serum. We noted that the pathogenic leptospire possessed a number of major and minor cross-reactive protein

antigens. The two nonpathogenic *Leptospira* spp. exhibited only a limited degree of cross-reactivity. These results confirmed the specificity of our radioimmunoprecipitation procedure and again pointed to the lack of genetic relatedness between these two groups.

An important goal of our study was to determine the cellular location of the major protein antigens of strain hardjoprajitno. We were particularly interested in those protein antigens present on the leptospiral cell surface, because the outer envelope has been shown to be a primary target for the antibody-complement bactericidal reaction (1) as well as a potent immunogen (2, 4-7, 11). Initially, we followed a simple fractionation procedure to separate the soluble and membrane-bound proteins of radiolabeled strain hardjoprajitno. We found that most of the major protein antigens were present in the membrane fraction. To determine which membrane proteins reside in the leptospiral outer envelope, we used a modification of the procedure described by Auran et al. (2) for the selective removal of the outer envelope. As expected, the outer envelope proteins represented a subset of the membrane proteins. We used a whole-cell radioimmunoprecipitation procedure to identify antibody-accessible proteins on the cell surface of intact, radiolabeled strain hardjoprajitno cells. Each of the nine protein antigens recognized by this procedure corresponded to a specific outer envelope protein.

Our results include the first reported identification and characterization of the protein antigens of the leptospiral outer envelope. Although Auran et al. (2) and others (4-7, 11) studied the immunogenic potency of the leptospiral outer envelope in animals, the antigens responsible for provoking a protective host immune response were not identified. Since surface-exposed outer envelope proteins are the only cellular proteins with direct access to the infected host, they may play a role in the pathogenesis of leptospirosis. Additionally, specific antibody directed against such proteins may be an important component of the host immune response. Studies are currently under way in our laboratory to determine whether sera from serovar *hardjo*-infected cattle will also recognize the same outer envelope protein antigens recognized by hyperimmune rabbit sera.

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